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NEW SURGICAL HORIZONS

From the genome to the proteome—biomarkers in colorectal cancer

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Abstract
Background and aims Colorectal cancer is the second leading cause of cancer-related death. Current clinical practice in colorectal cancer screening (fecal occult blood test, FOBT; colonoscopy) has contributed to a reduction of mortality. However, despite these screening programs, about 70% of carcinomas are detected at advanced tumor stages (UICC III/IV) presenting poor patient prognosis. Thus, innovative tools and methodologies for early cancer detection can directly result in improving patient survival rates.
Patients/methods Biomedical research has advanced rapidly in recent years with the availability of technologies such as global gene and protein expression profiling. Comprehensive tumor profiling has become a field of intensive research aiming at identifying biomarkers relevant for improved diagnostics and therapeutics.
Results In this paper, we report a comprehensive review of genomic, transcriptomic, and proteomic approaches for biomarker identification in tissue and blood with a main emphasis on two-dimensional gel-electrophoresis (2-DE) and mass spectrometry analyses.
Conclusion Proteomics-based technologies enable to distinguish the healthy patient from the tumor patient with high sensitivity and specificity and could greatly improve common classification systems and diagnostics. However, this progress has not yet been transferred from bench to bedside but could open the door to a more accurate and target specific personalized medicine with improved patient survival.

Keywords Colorectal cancer · Biomarkers · Genomics · Transcriptomics · Proteomics

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Introduction

Colorectal cancer is one of the most common malignancies in the world and the second leading cause of cancer-related death in the USA with an incidence of approximately 160,000 affected patients each year [37]. The current clinical practice in colorectal cancer detection with common screening methods (fecal occult blood test, FOBT; colonoscopy) has contributed to a reduction of mortality [45]. However, despite these screening programs, about 70% of carcinomas are detected at advanced tumor stages (UICC III/IV) presenting poor patient prognosis. Whereas the 5-year disease-free survival rate for early stage tumors (UICC stage I) exceeds 90%, this percentage is reduced to 63% in advanced stage carcinomas (UICC stage III) [55]. Therefore, detection of cancer at an early stage is critical for curative treatment interventions. Thus, utilization or application of innovative tools and methodologies for early cancer detection can directly result in improving patient survival rates.

In this context, biomedical research has advanced rapidly in recent years with the sequencing of the human genome and the availability of technologies such as global gene and protein expression profiling. Comprehensive tumor profiling has, therefore, become a field of intensive research aiming at identifying biomarkers relevant for improved diagnostics and therapeutics.

The term "proteome" was coined to describe a set of proteins that is encoded by a genome (proteins expressed by a genome). Proteomic research approaches issues that cannot be addressed by genome and transcriptome analyses alone. Thus, proteomics evaluates, e.g., protein abundance, posttranslational polypeptide modification, and protein–protein interaction, as well as functional and dynamic processes within the cell. Already today, proteomics—based on two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), and surface-enhanced laser desorption ionization (SELDI) technology—is able to distinguish the healthy patient from the tumor patient with high sensitivity and specificity, and will greatly improve common classification systems and diagnostics of the post-genome era [60]. However, this progress has not yet been transferred from bench to bedside but could open the door to a more accurate and target specific personalized medicine with improved patient survival.

Genomics in colorectal cancer

Aneuploidy

When the first quantitative measurements of the DNA content of cancer cells were performed, aneuploidy was defined as a variation in nuclear DNA content in the population of cancer cells. Since then, aneuploidy has been observed as a consistent genetic alteration of the cancer genome of different tumor entities [43].

Aneuploidy is commonly observed in colorectal cancer cells, especially in advanced stages. Saccani et al. analyzed 51 cases of normal mucosa adjacent to aneuploid tumors and showed a 7% incidence of aneuploidy, whereas the mucosa adjacent to diploid cancers demonstrated only diploid characteristics [64].

In addition, aneuploidy seems to precede the manifestation of malignancy: Löfberg et al. reported aneuploid biopsies in 25% of ulcerative colitis patients with a high risk for colorectal cancer development at least once during 10 years of observation. In other studies, aneuploidy has been repeatedly observed even in non-dysplastic mucosa of ulcerative colitis patients [29].

Despite the controversies, evidence suggests that a greater proportion of higher-stage tumors are aneuploid, with aneuploid tumors tending to have a higher growth rate and a poorer survival than diploid tumors [14]. A few studies report the prognostic value of tumor ploidy, especially in UICC stage II patients: Nori et al. compared the DNA content in patients with stage II disease and no evidence of relapse versus stage II patients with relapse. A total of 80% of patients with recurrence showed aneuploidy compared to only 40% of patients in the control group. Furthermore, aneuploidy was associated with a significantly higher tumor recurrence rate and a shorter overall survival [54]. Aneuploidy has also been observed in premalignant lesions [29]. Within those lesions, however, aneuploidy may be reversible over time once cells are no longer exposed to the inducing agent or carcinogen [56]. Thus, it is reasonable to suggest that genomic instability reflected by aneuploidy has to be followed by further cellular alterations to reach malignant properties. One of the decisive steps in this transformational process is the ability of genomically altered cells to proliferate, which is compulsory for clonal expansion [77]. Interestingly enough, immunohistochemical expression of the proliferation marker cyclin A was significantly correlated to aneuploidy in biopsies of patients with a subsequent carcinoma [29].

With increased resolution of cytogenetic techniques, such as chromosome banding, comparative genomic hybridization (CGH), spectral karyotyping (SKY), and multicolor fluorescence in situ hybridization, it became clear that, in addition, to nuclear aneuploidy, specific nonrandom chromosomal imbalances (heretofore referred to as chromosomal aneuploidy) exist [39]. Despite genetic instability in cancer genomes, cancer cell populations as a whole display a surprisingly conserved, tumor-specific pattern of genomic imbalances [40]. At early steps in the sequence of malignant transformation during human tumorigenesis, e.g., in pre-invasive dysplastic lesions, chromosomal aneuploidies can be the first detectable genetic aberration found [34]. This suggests that there are both an initial requirement for the acquisition of specific chromosomal aneuploidies and a requirement for the maintenance of these imbalances despite genomic and chromosomal instability. This would be consistent with continuous selective pressure to retain a specific pattern of chromosomal copy number changes in the majority of tumor cells [10]. The conservation of these tumor-specific patterns of chromosomal aneuploidies suggests that they play a fundamental biological role in tumorigenesis.

The progression of colorectal cancer is defined by the sequential acquisition of genetic alterations [21]. At the cytogenetic level, many of these aberrations can be visualized as specific chromosomal gains and losses. These aneuploidies result in a recurrent pattern of genomic imbalances, which is specific and conserved for these tumors [59]. For instance, one of the earliest acquired

genetic abnormalities during colorectal tumorigenesis are copy number gains of chromosome 7 [10]. These trisomies can already be observed in benign polyps, and can emerge in otherwise stable, diploid genomes. At later stages, e.g., in high-grade adenomas or in invasive carcinomas, additional specific cytogenetic abnormalities become common. Gains of chromosome 7 and chromosomal arms 8q, 13q, and 20q, and losses that map to 8p, 17p, and 18q have been published [50]. These chromosomal aneuploidies are accompanied by specific mutations in oncogenes and tumor suppressor genes, including ras, adenomatous polyposis coli (APC), and p53 [75]. Nowadays, it is common understanding that both the chromosomal aneuploidies and specific gene mutations are required for colorectal tumorigenesis (Fig. 1a,b).

Genetic biomarkers

Several genetic changes have been tested for their potential as biological markers in colorectal cancer surveillance. The evidence that colorectal cancer develops from normal mucosa via adenoma to carcinoma by accumulation of molecular alterations offers a large scale of opportunities in detecting stage-specific alterations at the DNA, RNA, and protein level.

Adenomatous polyposis coli

The APC gene regulates proliferation and cellular adhesion, and is well known as the tumor-suppressor gene that prevents colorectal carcinogenesis. Mutations of the APC

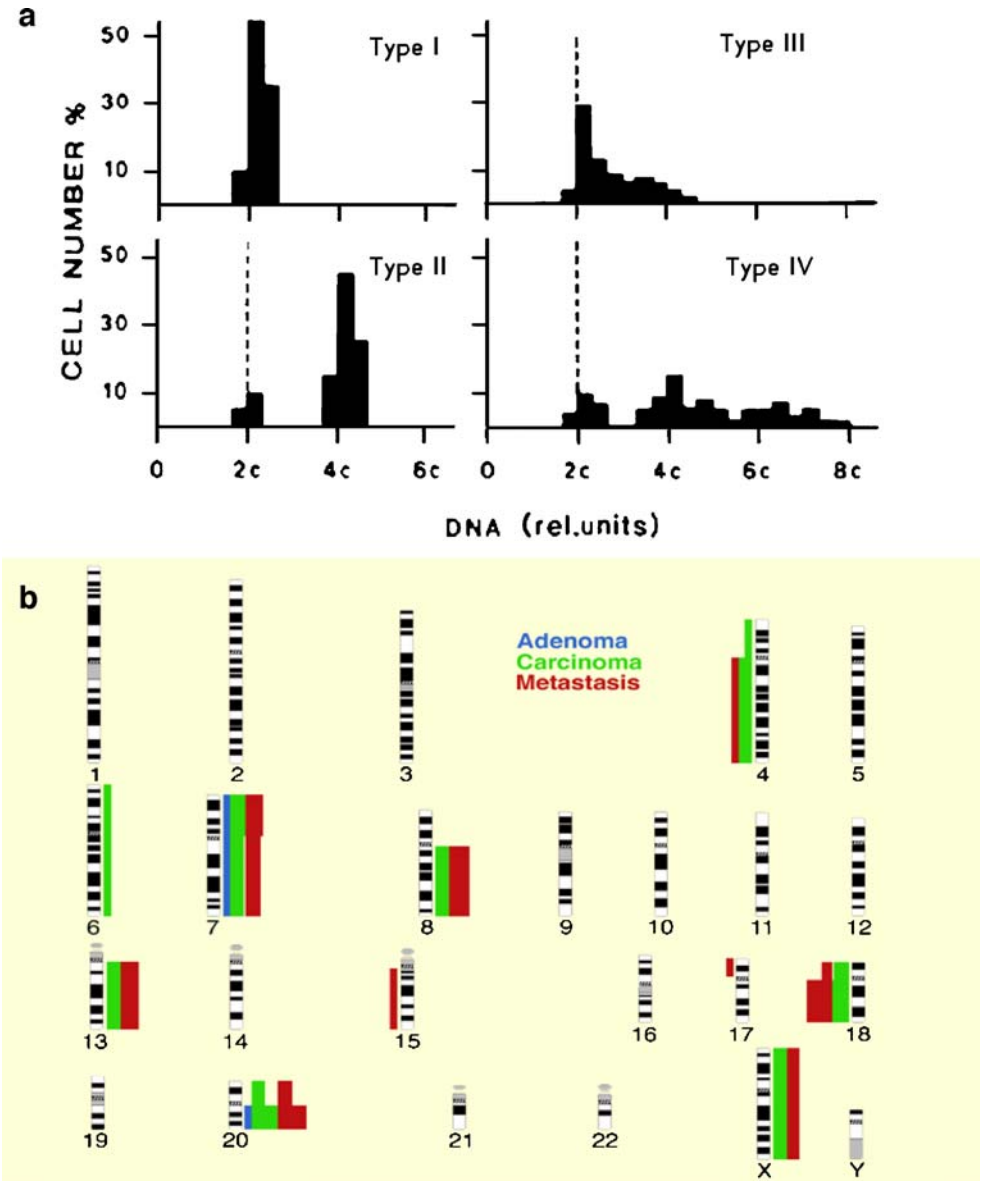

Fig. 1 **a** Genomic instability is reflected by DNA histograms that were classified according to Auer. Histograms of types I, II, and III characterize euploid cell populations, whereas type IV reflects an aneuploid population of interphase nuclei with decreased genomic stability. **b** Chromosomal instability assessed by comparative genomic hybridization (*CGH*). Summary of DNA copy number changes in 12 adenomas, 16 primary colorectal carcinomas, and 12 liver metastasis. *Bars on the left side of the chromosome ideogram* denote a loss; *bars on the right side* denote a gain of sequence in the tumor genome. The number of alterations has been normalized to ten cases per stage progression group

gene are considered to be involved in about 60 to 80% of sporadic colorectal cancers. Germline mutations of APC cause the familial adenomatous polyposis syndrome (FAP). The inactivation of this tumor suppressor gene, located on Chromosome 5q21, is recognized as an early event in colorectal carcinogenesis. However, it has been proven that screening for APC mutations has no value in diagnostics or surveillance for sporadic colorectal cancer [20].

K-RAS

K-ras is a proto-oncogene, which adheres to the inner face of the cell membrane. It is the most frequently mutated gene of the RAS family (K-, H-, and N-ras) with mutations found in approximately 50% of colorectal adenomas larger than 1 cm in size as well as in 40–50% of colorectal carcinomas, especially when codon 12 is involved in the mutation [35]. The RASCAL study published by Andreyev et al. is the largest survey on this issue. It comprises a collection of data achieved by groups from 13 countries concerning the question of the prognostic importance of k-ras mutation. The study reports an increased risk of recurrence and death associated with k-ras mutations. Furthermore, they stated that some mutations could be more aggressive than others [7]. Other surveys report worsened prognosis associated with mutations. The presence of mutant ras may also predict poor response to chemotherapy [12]. The high frequency of mutations and its early appearance in colorectal cancer development points to its potential in serving as a biomarker. Assays for detection of ras mutations in stool samples have already been developed but have not yet been implemented into clinical application [69].

Microsatellite instability and mismatch repair genes

Like in hereditary non-polyposis colon cancer (HNPCC), mutations in the DNA mismatch repair genes are also present in 15–20% of patients with sporadic colorectal cancer. This defect in DNA repair causes genetic microsatellite instability (MSI). Whether or not MSI could be an independent prognostic factor was investigated by several studies. Halling et al. performed a retrospective study that included more than 500 patients and found a decrease in 5-year survival in 74% of UICC stage III patients with MSI as compared to 55% of patients without MSI [32]. However, Watanabe et al. could not observe statistically significant survival differences between these two groups [79].

P53

Located on the short arm of chromosome 17, p53 is regarded as one of the most important tumor suppressor genes, with a number of key functions, such as initiation of apoptosis, DNA damage repair, and cell cycle control [76]. Mutation and loss of heterozygosity of p53 appear to arise most frequently during the transition from adenoma to carcinoma. These events result in expression of an abnormal p53 protein that accumulates in the colonocyte nucleus and can be detected immunohistochemically. Several studies report a worse tumor prognosis and significantly shorter survival time correlated to p53 mutation [41]. In addition, Ahnen et al. [3] showed a worse 5-year survival rate in patients with increased p53 expression levels after 5-FU chemotherapy. However, these data could not be confirmed by Watanabe et al. [79], who did not find a correlation of p53 immunopositivity with either prognosis or response to chemotherapy.

CEA

First described in 1965, the carcinoembryonic antigen (CEA), a highly glycosylated protein, has become a routinely assessed marker for colorectal cancer [27]. Low costs and easy access of CEA assays compared to other diagnostic methods such as CT, MRI, and colonoscopy made it especially attractive. Several studies reported that CEA is an independent prognostic factor [70]. Harrison and coworkers reviewed nearly 600 patients who underwent surgery for node negative colorectal cancer. The preoperative CEA level and the disease stage predicted survival by both univariate and multivariate analysis [33]. In another study, Chapman et al. reported that, although the 5-year survival rate for patients with an increased CEA was 39 versus 57% for patients with normal CEA levels, the proportion of patients with an elevated CEA level increased with more advanced tumor stage and poorly differentiated tumor grade [13]. However, Moertel et al. [51] reported that, among patients with recurrence, only 59% showed elevated CEA levels in serum, and 16% were false positive. Fletcher et al. showed that CEA screening would yield a sensitivity of 30–40% and a specificity of 87%. Based on these numbers, they calculated that, for every single colorectal cancer patient detected by CEA, there would be 250 false positive patients and 60% of all cancers would be missed [23].

Data for the detection of metastasis by CEA testing are likewise disappointing. Glover et al. [26] could prove the evidence of liver metastasis in only 33%, and Mori et al. [52] published a detection rate of 6% for pulmonary metastasis using CEA.

In conclusion, CEA screening is hampered by low sensitivity and specificity, and leads to a large number of false positive results. Nevertheless, CEA has proven to be beneficial as a surveillance marker after curative

resection, especially if CEA levels were decreasing post-operatively [13].

DNA-based stool markers and FOBT

The challenge for DNA stool testing involves separating abnormal human DNA from both bacterial DNA and normal human DNA in the stool, amplifying it, and testing it for genetic abnormalities [8]. One advantage is represented by the fact that DNA fragments from the stool of colorectal cancer patients have been shown to have an even higher integrity than stool DNA of healthy patients [11]. The heterogeneity of mutations in colorectal cancer has lead to testing of multiple genetic targets. First, K-ras mutations were discovered in stool of patients with colorectal carcinomas and other malignancies harboring K-ras mutations. Sidransky et al. [69] reported K-ras mutations in eight of nine patients with colorectal carcinomas in both stool and tumor tissue. Because mutant K-ras is expressed in less than half of all colorectal carcinomas, early studies found poor sensitivity for detecting K-ras mutations in stool of colorectal cancer patients [2].

Dong et al. [18] reported mutations of stool DNA in 71% of colorectal cancer patients by using K-ras, p53, and BAT-26 as screening targets. These findings could be validated by Ahlquist and coworkers, who tested stool DNA for the same targets. In addition, they searched for APC mutations and long DNA, a marker for non-apoptotic shedding of epithelial colonocytes. They detected DNA mutations in stool in 91% of the cancer patients and in 82% of patients with adenomas smaller than 1 cm [2].

Within over 4,400 asymptomatic patients undergoing colonoscopy, Imperiale et al. compared fecal DNA testing to FOBT using mutation analysis for K-ras, APC, p53, BAT-26, and long DNA. They could detect 52% of the diagnosed carcinomas by fecal DNA testing as compared to 13% with FOBT using three cards. For advanced adenomas, fecal DNA testing identified 15% and FOBT 11%. Specificity was equal with 94%, respectively [36].

As recommended by the American Gastroenterological Association, FOBT has been introduced as a major screening test for colorectal cancer. Based on three large randomized clinical trials, screening with FOBT was able to reduce mortality of colorectal cancer up to 33% in the annually tested FOBT group and up to 21% in the biannually tested one [46]. Despite these findings, false negative results have been published in 20–30% of colorectal cancer patients [66]. However, the use of FOBT as a biomarker has inherent flaws. Cancers may bleed intermittently; in addition, the majority of gastrointestinal bleeding occurs from cause other than cancer. Thus, specificity of FOBT is limited, and improving sensitivity further compromises specificity.

Transcriptomics

Methodology to analyze gene expression of tumor genomes on a high-throughput scale has become available through the development of microarray-based gene expression profiling. This method has been first described by Schena et al. [65] and enables the simultaneous analysis of thousands of genes.

To elucidate how genomic imbalances affect chromosome-specific gene expression patterns, Upender et al. [73] used an experimental model system in which the only genetic alteration between parental and derived cell lines is an extra copy of a single chromosome. Several important conclusions can be drawn from that analysis: First, regardless of chromosome or cell type, chromosomal trisomies result in a significant increase in the average transcriptional activity of the trisomic chromosome. In addition, aneuploidy not only affects gene expression levels on the chromosomes present in increased copy numbers, but a substantial number of genes residing on other chromosomes significantly increased or decreased, apparently in a stochastic manner. This observation is of course consistent with known mechanisms of gene regulation (e.g., activator and suppressor proteins, signaling pathways) and the fact that genes residing in a given pathway are, for the most part, distributed throughout the genome on different chromosomes. The consequences of constitutional chromosomal trisomies on transcriptional activity have also been analyzed in non-cancerous fetal cells and attained similar conclusions [47]. These studies concluded that the average gene expression of trisomic chromosomes is clearly increased and that expression levels of multiple genes throughout the genome were dysregulated. Analogous results could be obtained in primary colorectal tumors: We found that specific and recurrent chromosomal aneuploidies exert strong and direct influence on gene expression levels of resident genes on the affected chromosomes by analyzing tissue samples from 36 patients with sporadic colorectal carcinoma. In addition, increasing genomic instability, aneuploidy, and a recurrent pattern of chromosomal aberrations are accompanied by distinct gene and protein expression patterns that correlate with subsequent stages of colorectal cancer progression [30].

This global dysregulation of the transcriptome and proteome of cancers of epithelial origin may also reflect on our ability for therapeutic intervention: Although the consequences of a simple chromosomal translocation, such as the *BCR/ABL* fusion in chronic myelogenic leukemia, can be successfully targeted with an inhibitor of the resulting tyrosine kinase activity such as Gleevec® (Novartis), the normalization of the complex dysregulation of transcriptional activity in carcinomas requires a more general, less specific, and hence, more complex interference [19]. In this context, biomarker search for colorectal cancer by means of

microarray expression profiling has been performed by many groups. For a comprehensive overview, see Shih et al. [68]. However, the comparison of the often-different results proofed to be difficult because of, e.g., the different array platforms used. So far, no identified gene expression markers have been implemented for clinical application yet.

Proteomics in colorectal cancer

Whereas DNA acts like a "blueprint," proteins are the dynamic components of the cell. Neither the existence of a given DNA sequence nor the RNA expression allows the prediction of the synthesis of a corresponding protein. Furthermore, the structure, function, and cellular location of proteins is not described sufficiently by DNA or mRNA sequence. Examination of the proteome allows also for detection of functional important post-translational modifications such as glycosylation and phosphorylation, which may not be seen at the genome level.

The term proteome was first defined in 1994 and denotes the entirety of proteins expressed by the genome. Proteomics techniques have rapidly evolved and are now widely applied to monitor disease specific alterations [49].

Techniques in cancer proteomics

Laser-capture micro-dissection (LCM)

The ideal method to dissect the cells of interest from inhomogeneous tissue is laser-capture micro-dissection (LCM). The new computerized LCM equipment is able to isolate normal, precancerous, and cancer cells — even of the same specimen — without denaturing them by the laser energy applied. This allows for intra- as well as for inter-individual protein expression pattern comparison [17]. Despite the advanced technology, LCM has to be performed by an experienced pathologist, is still time consuming, and the derived protein yield is low. Therefore, its use in large trials is rather difficult.

Two-dimensional gel electrophoresis (2-DE)

In proteome research, 2-DE is still the cornerstone separation technique for complex protein mixtures. The method is based on two biophysical principles, which are used in the first and second dimension: In the first dimension, all proteins are characterized by their differentially charged amino acid residues. The isoelectric point of a protein is the specific pH value at which the sum of negatively and positively charged amino acid residues is equal. At this point, the protein stops its migration within the electric field [24]. This step is followed by the protein separation within the second dimension: The different size and molecular weight of the separated proteins result in a different migration speed within the gel. Since the 2-DE technique has been implemented by O'Farrel and Klose in 1975, it has experienced a tremendous development, such as the invention of IPG strips for different pH ranges that warrant higher reproducibility. This invention has constituted the breakthrough of the 2-DE-based proteome research [28]. To visualize the separated protein spots, the two-dimensional gels are stained using silver, comassie, or sypro ruby. Images are scanned and digitally compared using different software available. These are offering automated spot-detecting and spot-matching functions, and partly, have integrated statistical software packages. These techniques enable one to highlight proteins that are differentially abundant in one state versus another (e.g., tumor versus normal; Fig. 2).

2-D DIGE

For high throughput proteomic studies, the image analysis can be considered a "bottleneck." Protein samples are separated on individual gels, stained, and quantified in conventional 2-D gel electrophoresis, followed by computer-assisted image comparison and analysis. Because the multistep 2-DE technology often prohibits different images from being perfectly super imposable, image analysis is very time consuming. The development of the DIGE technique by Ünlü et al. addresses this problem. Here, two samples are labeled in vitro using two different fluorescent cyanine minimal dyes that are differing in their emission and excitation wavelength. Samples are mixed before IEF and separated on a single 2-DE gel simultaneously [72]. Images are then overlaid and subtracted after consecutive excitation with both wavelengths, whereby, only differences between the two samples are visualized.

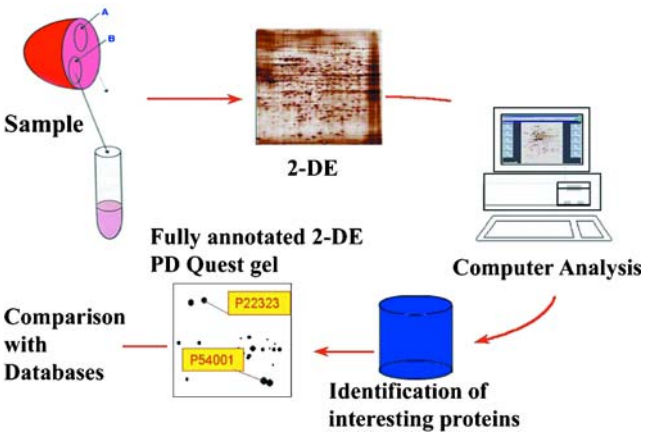

Fig. 2 Algorithm of the 2-DE approach from sample preparation to mass spectrometry

Matrix-assisted laser desorption ionization MS

Within the matrix-assisted laser desorption ionization (MALDI) technique, matrix and sample are co-crystallized on the MALDI plate and irradiated with a laser pulse [74]. The matrix absorbs the energy and acts as an intermediary for the co-desorption and ionization of sample and matrix. The ions are accelerated in an electrical field and enter a field-free drift tube. The mass-related time of flight is detected, and the analogue signal is converted and digitalized. The experimentally generated masses are compared to a set of mass profiles in a protein database, e.g., SwissProt, ExPASy, or UniProt. The most similar pattern determines the protein "hit." The tighter the mass tolerance, the more stringent is the identification (Fig. 3a–c).

SELDI and protein-chip technology

A particular promising technique for proteome screening is based on SELDI time-of-flight (SELDI-TOF) MS. A major advantage of SELDI is that complex protein mixtures (e.g., serum or cell extracts) can be directly analyzed by MS without any prior separation and purification. SELDI-TOF utilizes chromatographic surfaces that retain proteins from a complex sample mixture according to their specific properties (e.g., hydrophobicity and charge), with the molecular weights of the retained proteins then being measured by TOF MS [16]. The mass spectra patterns obtained reflect the protein and peptide contents of the samples. Protein identification itself needs to be performed in an additional analysis step [67]. The reliability and reproducibility have been proven even if variation coefficients of 8–10% indicate the need for technical repeats [67]. However, specific caution has to be taken when performing bioinformatics analysis of SELDI-based protein pattern [58]. SELDI-TOF MS is particularly well suited to evaluate low-molecular proteins (0.5–25 kDa) and is, as such, complementary to the 2-DE approach. Looking at patients suffering from e.g., breast, prostate, and colorectal cancer, SELDI-based protein profiling was able to distinguish healthy patients from tumor patients by class prediction with high sensitivity and specificity [15, 31, 44, 57]. Unfortunately, most of the SELDI-based studies remained at the level of defining SELDI-based protein profiles and missed to subsequently identify the protein features that would then allow the application of validated makers into clinical application.

Biomarkers in tissue proteomics

All techniques mentioned above have revolutionized the ability to characterize the proteome in human tissue. Although we are at the very beginning of identifying proteins as reliable biomarkers for cancer screening, the number of publications that report promising results on this issue has risen tremendously during the last years.

Jungblut and coworkers were among the first to report polypeptide changes that are associated with malignant

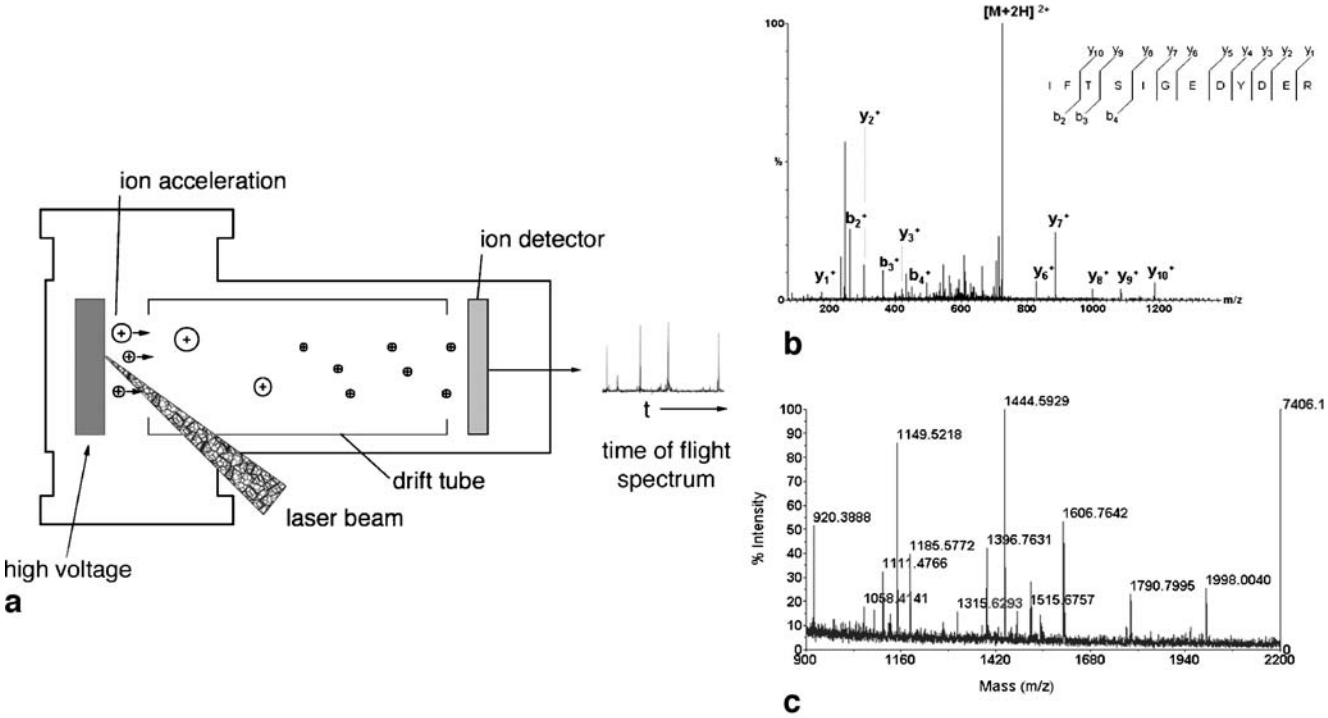

Fig. 3 **a** MALDI scheme, **b** and **c** MALDI and MS/MS spectra for identification of vimentin

transformation of the colon mucosa. Running 2-DE in 15 patient samples with colorectal carcinomas and 13 samples of healthy individuals, they detected one protein with a molecular mass of 13 kDa that was significantly overexpressed in tumor tissue. This protein, identified as calgranulin B, was up regulated in 13 of 15 carcinoma samples (87%). Furthermore, they could verify the presence of calgranulin B in precancerous lesions [38]. These findings confirmed earlier results of Roseth et al. who could describe elevated levels of the heterodimeric protein calprotectin composed of calgranulin B and A in stool samples of patients with gastrointestinal carcinomas [63]. Nevertheless, the exact function of calgranulin B and its role in the process of malignant transformation still needs to be clarified.

A similar approach was used by Friedman and coworkers who used 2-D DIGE coupled with MS to investigate tumor-specific changes in the proteome of colorectal cancer and adjacent normal mucosa. With matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and tandem MS (TOF/TOF), they could identify 52 individual proteins that showed significant expression differences between colorectal cancer and normal mucosa [25].

To identify specific protein markers for early detection of colorectal cancer, Alfonso et al. performed 2D-DIGE coupled with MS. They analyzed tissue samples from seven patients with colorectal cancer and adjacent normal mucosa. Protein expression was compared between each tumor and normal-paired mucosa tissue. They could detect differences in abundance of 52 proteins with statistical variance of the tumor versus normal-spot volume ratio within the 95th confidence level ($p<0.05$). MALDI-TOF coupled with database interrogation could then identify 41 of the 52 proteins that were differentially expressed. The proteins found were mainly involved in regulation of transcription (metastasis-associated protein 1, synovial sarcoma X5 protein), cell communication and signal transduction (annexins IV and V, relaxin, APC), cellular reorganization and cytoskeleton (vimentin, cytokeratins, ß-actin), and protein synthesis and folding (heat shock protein 60, cathepsin D, RSP4, calreticulin). Other proteins such as MTA-1, SSX5, and dynein have not been described previously to be expressed in colorectal cancer [5].

In analogy to the Vogelstein model of colorectal carcinogenesis, we performed a detailed analysis to identify sequential alterations of the proteome that defines the transformation of normal epithelium and the progression from adenomas to invasive disease. We have analyzed tissue samples from 15 patients, including the mucosa–adenoma–carcinoma sequence from individual patients. We determined the degree of genomic instability during carcinogenesis by measuring DNA contents and assessed protein expression levels by means of 2-DE and subsequent MS. Two-dimensional gel electrophoresis revealed a total of 112 polypeptide spots that showed an at-least-twofold differential expression between the four stages of carcinogenesis. A total of 72 of these polypeptides could be characterized by MS, and 46 of those were exclusively overexpressed in tumors and metastases. Unsupervised principal component analysis allowed separation of adenomas, carcinomas, and metastases based on protein expression profiles. Interestingly, two dysplastic polyp samples did not conformingly cluster in their cohort and were closer to the malignant samples. Both polyps revealed aneuploid DNA distribution patterns, indicating an increased malignancy potential [61] (Fig. 4).

In addition, Kwong et al. performed a study that assessed both gene and protein expression in parallel across-progressive stages of colorectal cancer (ten samples of each: normal mucosa, adenoma, Duke B, Duke C, Duke D, liver metastasis). All samples were microdissected followed by protein and RNA extraction from the same frozen tissue block. 2D-PAGE and complementary DNA (cDNA) microarray analysis could demonstrate that, despite minimal overlap of the proteins and genes accessible by these technologies, both lead to consistent, complementary conclusion regarding the nature and progression of disease [42].

Although the implementation of cancer proteomics-based findings into clinical routine is still at the very

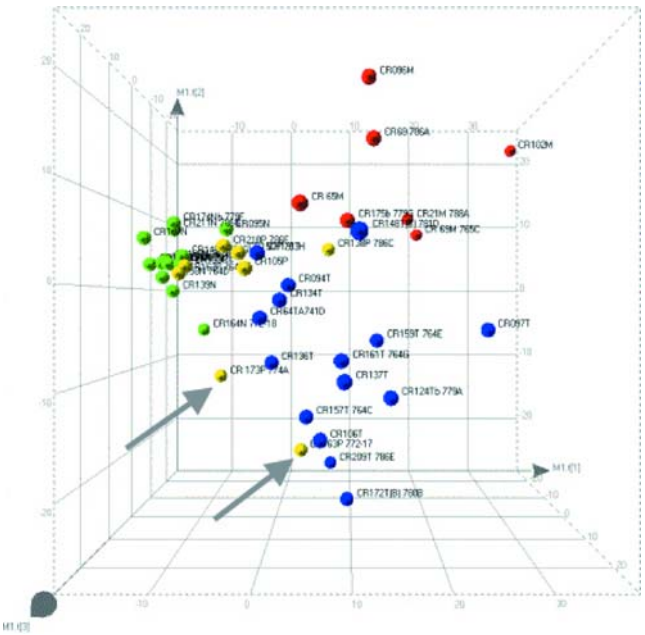

Fig. 4 PCA plot of the polypeptide expression with the normal samples (*green*), polyps (*yellow*), cancer (*blue*) and metastases (*red*). The arrow-marked polyp samples showed closer cluster to the cancer probes reflecting their aneuploidy in the DNA measurement

beginning, first reports reflect their clinical potential: Roblick et al. investigated a case of a poor differentiated malignant pelvic mass involving the left ovary and the rectosigmoid colon, which could not be identified by standard histopathological and immunohistochemical methods to be of colonic or ovarian origin. After ruling out other possible origins by CT scan and MRI, ten colon cancer samples and ten high-grade ovarian carcinoma samples were run with 2-DE and matched against the pelvic tumor. The image analysis could clarify the tumor being of colorectal origin, so that the appropriate adjuvant therapy could be performed [62].

Such findings are well in line with data published by Nishizuka and Chen, which developed a multistep protocol using 60 human cancer cell lines from the National Cancer Institute (NIH), Bethesda, to discriminate colon and ovarian cancer by genomic and proteomic approaches. The steps included identification of candidate markers using cDNA microarrays, verification of clone identification by re-sequencing, corroboration of transcript levels using affymetrix oligonuctleotide chips, quantification of protein expression by reverse phase protein micro-array, and prospective validation of candidate markers on clinical tumor sections in tissue microarrays. They could identify villin as a marker for colon cancer cells and moesin for ovarian cancer cells [53].

A further report by Allal et al. underlines the clinical value of proteomic research. They separated proteins from tissue samples from patients with locally advanced rectal tumors before radiotherapy by narrow pH-range 2-DE and could demonstrate that the expression of the following proteins identified by MALDI-TOF correlated with radio-resistance (tropomodulin, heat shock protein 42, β-tubulin, annexin V, calsenilin) or radiosensitivity (keratin type I, notch 2 protein homolog, DNA repair protein RAD51L3) [6].

A more genetic approach was published by Wang and his colleagues, who could predict the recurrence of Dukes B colon cancer with an overall accuracy of 78% using an affymetrix U133a GeneChip containing approximately 22,000 transcripts. They analyzed 74 patients with Dukes B colon cancer and could predict recurrence by using a micro-array-based 23-gene expression signature in 13 out of 18 patients correctly. These findings could lead to the conclusion that, if validated in a larger population, patients with Dukes B colon cancer and a high predicted risk of relapse should be upstaged to receive adjuvant therapy [77].

Biomarkers in serum proteomics

The ideal biomarker for colorectal cancer would be detectable in a readily accessible body fluid, such as blood, and would reflect not only presence of disease but the status of the disease process as it changes over time [22]. Improvement of disease detection could be made by using single-protein biomarkers to screen for malignancy, as it has been the case for prostate-specific antigen in prostate cancer. However, it is more likely that only a panel of different biological markers will guide to success, when high sensitivity and specificity should be reached. However, despite extensive research efforts, the identification of robust biomarkers and/or their implementation into clinical routine for colorectal cancer screening has been disappointing so far.

Nevertheless, important progress has been achieved in characterizing the serum proteome during the past years as follows [71].

The implementation of the SELDI technique disembogued in various protein expression patterns for the most common tumor entities such as breast-, ovarian-, prostate-, and colorectal cancer. Several proteins were found to be up regulated in the serum of tumor patients [1, 9, 80].

Yu and colleagues presented a trial with serum samples of 182 patients including 55 with colorectal cancer of various stages (Dukes A-D), 35 with colorectal adenomas, and 92 healthy individuals. Using SELDI-MS combined with a sophisticated bioinformatics tool, they could differentiate patients with colorectal carcinomas and those with adenomas with a specificity of 83%, a sensitivity of 89%, and a positive predictive value of 89% [80]. This analysis was based on class prediction analysis by SELDI-based pattern recognition and lacked subsequent identification and validation of the discriminative peaks/features.

An approach with SELDI-TOF/MS was done by Albrethsen and coworkers who compared the protein profiles of colon cancer serum with serum from healthy patients and the protein profiles of colon tumors with normal colon mucosa. They could show that the expression of human neutrophil peptides (HNP)-1, HNP-2 and HNP-3, also known as α-defensin-1, α-defensin-2, α-defensin-3, is up regulated in the tumor microenvironment, as compared to normal colon tissue. They also could determine that, in serum samples of patients with colorectal carcinomas, HNP 1–3 concentrations are elevated, as compared to the serum of the healthy control group [4]. These data are in accordance with a nearly simultaneously published trial of Melle et al. [48].

In addition, Ward et al. as well as our own group, were able to define markers that distinguish colorectal cancer sera from serum samples of healthy individuals with high sensitivity and specificity. SELDI data were validated using specific immunoassays (enzyme-linked immunosorbent assay, ELISA), and both studies found C3a-anaphylatoxin to be the marker with the highest diagnostic potential.

In our trial, the evaluation of the C3a–desArg levels using ELISA was able to predict the presence of colorectal cancer with 96.8% sensitivity and 96.2% specificity. The

fact that C3a–desArg levels were elevated in 86.1% of serum samples from patients with colorectal adenomas underlines its value and potential as a diagnostic marker [31, 78].

Summary and future perspectives

Genomic aneuploidy in colorectal cancer correlates with specific chromosomal gains and losses. The predominance of specific chromosomal aneuploidies in colorectal cancers also affects the transcriptome of cancer cells. In addition, increasing genomic instability and a recurrent pattern of chromosomal aberrations are accompanied by distinct protein expression patterns that correlate with subsequent stages of colorectal cancer progression. Identified proteins undergo extensive posttranslational modifications, thus, multiplying the transcriptional dysregulation. The employment of comprehensive gene and protein expression profiling in subsequent stages of colorectal cancer progression, thus, allowed the identification of genes and proteins that now warrant further validation by, e.g., RNA interference (RNAi) experiments, to prove their potential for gene- and protein-expression-tailored individualized diagnostic, prognostic, and therapeutic approaches.

In the future, clinical medicine will integrate proteomic and genomic knowledge. This transfer from "bench to bedside" will open up new diagnostic and prognostic potentials for pathology, clinical routine, as well as for patient surveillance, and define novel therapeutic targets. Early diagnosis for malignant disease will be feasible from easily accessible body fluids using chip technology, new inventions in the field of MS, as well as multiplex immunoassays. Tissue biopsies will still be evaluated by routine histopathology, but further on, pathologists will be able to use proteomic and genomic techniques to subclassify tumor entities and predict individual prognosis, which includes response prediction to radio- and chemotherapy, respectively.

What is the surgeon's role in such scenario? The surgeon guides the multidisciplinary treatment of patients in most cases, and thus, the surgeon builds the necessary bridge between cancer patient and proteomic and genomic research. Current and future research will rely on the surgeon's ability to collect representative material, which must be correlated with clinical outcome and response to treatment. It is the surgeon who must organize the logistic pathway, from obtaining a patients written consent to sample collection in the operating room, and delivery to the pathologist and the proteomic laboratory. He or she must guarantee efficient sampling with short ischemic times to avoid protein denaturation. In addition, a major role for the surgeon within the field of clinical proteomics lies in focusing the basic scientist on clinical relevant questions.

Efficient proteome and genome research is only possible in a multi-disciplinary team consisting of surgery, pathology, molecular biology and biophysics/chemistry, as well as bioinformatics. Such collaboration will, in the future, become the basis of proteomic and genomic centers.

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